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Blackfly Larvicide Efficacy, Bacterial Distribution and Regeneration of Bacillus Thuringiensis Ser. H14 (BTH-14) in a 10 Kilometre Segment of the South Saskatchewan River

Conducted by G. Khachatourians

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ANNUAL REPORT

Blackfly larvicide efficacy, bacterial distribution and regeneration of *Bacillus thuringiensis* ser. H14 (BTH-14) in a 10 kilometer segment of the South Saskatchewan River.

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G.G. KHACHATOURIANS

Bioinsecticide Research Laboratory
Department of Applied Microbiology & Food Science
College of Agriculture
University of Saskatchewan
Saskatoon, SK. S7N 0W0

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Project title: Blackfly larvicide efficacy, bacterial distribution and regeneration of *Bacillus thuringiensis* ser. H14 (BTH-14) in a 10 kilometer segment of the South Saskatchewan River.

A. Summary

1. Executive summary

The purpose of this study was to examine some underlying problems that were deemed to affect the South Saskatchewan River Blackfly Abatement Program in Prince Albert Region. This study was investigated in five objectives.

i) Examination of naturally occurring aquatic bacteria. We found that there is a diverse number of bacteria, reaching densities as high as 2 x 10^4 /ml (20 million bacteria per liter) in the South Saskatchewan river, during the latter part of August. There is also a variation in the density of these bacteria and this probably relates to the various physical parameters of the river such as depth, flow rate and surrounding vegetation. We found that vegetation alone vary significantly across the 10 km range under our study. Bacteria which produce extracellular proteolytic enzymes have been detected in the river samples. The number of *B. thuringiensis* colonies at the Birch Hills ferry site, before application of BTH-14 ranged from 0-1.5 x 10^3/ml.

(ii) Distribution of BTH-14 spores, cells and crystal toxin in the river. The density of *B. thuringiensis* downstream from the application site,

increased, overall, 3 hr post-application of BTH-14. After this time period, the density of *B. thuringiensis* declined. There was considerable variation in *B. thuringiensis* density at the various sampling stations and again this probably reflects the heterogeous nature of the physical aspects of a river and its ecosystem. We could not assess the distribution of the crystal toxin immediately after the spray, as this was rapidly diluted below detection limits of laboratory bioassays .

(iii) To determine blackfly larva associated bacteria and BTH-14 induced mortality. Two main species of blackfly larvae were identified in the South Saskatchewan River at, and downstream from, the Birch Hills ferry site, *Simulium luggari* and *S. vittatum*. Forty-eight hours after application of BTH-14, a 7.75- and 4.69-fold decrease, in *S. luggari* and *S. vittatum* larvae, respectively, was observed. This represents a reduction in *S. luggari* and *S. vittatum* larval population by 87.1% and 78.7%, respectively, after BTH-14 application. The mortality of blackflies was observed up to 10 km downstream from the application site. Microbiological variables which may interfere with BTH-14 efficacy were examined. The proteolytic bacteria found in the South Saskatchewan River inspite of their proteases did not interfere with the in vitro efficacy of BTH-14 in laboratory assays. Whether or not this observation substantiates that which occurs in situ remains to be demonstrated. We did not find evidence for midgut bacteria of blackfly larva to antagonize the effects of BTH-14.

(iv) Examination of the recycling potential in BTH-14 treated blackfly larvae. Our data shows that in the laboratory BTH-14 can be successfully recycled and cause mortality in larvae. Previous to our application of BTH-

14, we observed a standing population of *B. thuringiensis* in the South Saskatchewan river. The origin of this *B. thuringiensis* may be either an endemic bacterial species or an introduced species from the recycling of previous BTH-14 applications in earlier months of this summer. In either case, this population may contribute to blackfly larvae mortality as the *B. thuringiensis* is being recycled. To further elucidate this relationship, the *B. thuringiensis* and blackfly larval populations need to be carefully monitored in non-application stations to determine whether increases in population densities of blackfly larvae are followed by epizootics caused by *B. thuringiensis*.

2. Lay language summary

Bacillus thuringiensis ser. H14 (BTH-14) is a biological control agent which specifically kills the larvae of mosquitoe and blackfly species. Commercial formulation of BTH-14 has been applied for the recent years into the South Saskatchewan River near Prince Albert (Birch Hills ferry site) where blackflies cause considerable disturbance to livestock. The Provincial Blackfly Abatement program results in partial control and a success rate of 60-75%. In this project the mortality of blackfly larvae was monitored up to 10 km downstream of the application site. A significant decrease in larvae of two species of blackfly was observed within 48 hours of application over all 5 stations spanning 10 km downstream of the BTH-14 application station on the river. In analyzing the post-application distribution of BTH-14 active ingredients, it was found that cells, spore, and toxic crystals are rapidly moved from the spray station along the river as well as into various depths of the water. Some of this material was found to be associated with the river vegetation and sediments thus affecting full

delivery of BTH-14 to targets. In addition, certain naturally occurring river bacteria were found which produce protein degrading enzymes, which in the lab condition did not interfere with the larvicidal toxin protein of BTH-14. Larvae dead from BTH-14 served as a recycling reservoir for limited growth of BTH-14 cells.

B. Technical Report

1. Abstract

BTH-14 (66.2 liters; Teknar, Lot No. 1140549, Zoecon, Dallas, Texas) was applied to the South Saskatchewan River (Birch Hills ferry station) to a concentration of 1.5 ppm. Downstream from the application station, were 5 predetermined sampling stations. The dilution rate of BTH-14 after application, was assessed. The number of blackfly larvae before and after BTH-14 application were monitored. Furthermore, naturally occurring bacteria in the river which may antagonize the effects of BTH-14, and the gut microflora of live and dead blackfly larvae, were evaluated.

Two blackfly species, *Simulium luggari* and *S. vittatum*, were the most prevalent at all the sample stations. Differences in larval mortality were also correlated to the instar stage of each of the two blackfly species. Considerable blackfly larval death occurred within 48 hours of BTH-14 application and within 10 km of the point of application. The concentration of BTH-14 decreased slightly further away from the point of application. Horizontal and vertical stratification of BTH-14 was observed at some sample stations. At stations containing vegetation, rapids or rocks, additional changes in BTH-14 sample distribution was observed.

Samples from the South Saskatchewan River showed that at least 4 types of proteolytic bacteria were found as part of the natural microbiota.

However, these bacteria did not antagonize the effects of BTH-14 in laboratory tests.

Samples of dead blackfly larvae, 48 h post-treatment, were found to harbour BTH-14. When these bacteria were subcultured and a bioassay was performed on blackfly larvae, 100% mortality occurred within 12 hours. Midgut bacteria were isolated from dead blackflies before our BTH-14 application. Some of these bacteria were BTH-14-like and subsequent subculturing and bioassay resulted in 100% mortality within 48 hours. This suggests that BTH-14 is (1) endemic to the South Saskatchewan River or (2) has become established, to some extent, due to previous applications.

2. Background and objectives.

Female adult blackflies are blood-feeding insects and as such can be an annoyance to livestock so that economic losses may be apparent. The annoyance that blackflies cause to livestock may be exemplified in the interuption of livestock breeding cycles, reduction in livestock weight gain and milk reduction. The larvae of certain economically important blackfly species prefer fast-flowing rivers such as the South Saskatchewan River near Prince Albert, Saskatchewan. Coupled with the fact that many farms in that area have livestock, a blackfly problem exists.

Until recently a chemical insecticide, methoxychlor, was used to kill blackfly larvae in Saskatchewan. However, due to environmental concerns, the use of methoxychlor has been restricted by Pesticide Directorate. The biological control agent, *Bacillus thuringiensis ser. H14* (BTH-14) is very effective in killing blackfly larvae if the environmental parameters of the application stations are known. If such environmental details are unavailable BTH-14 has a limited values in complete control of population

killing. At optimal dosage, effectiveness of kill could range from 20-75%. Commercial formulations of this bioinsecticide are readily available, and control efforts are undertaken for direct administration of the formulation into the river. However, due to the specific nature of each river structure, depth and flow rate, temperature and suspended material as well as biotic factors, the application time, rate and type must be continuously refined so as to produce the most effective kill of blackfly larvae. For example, many of these parameters downstream from the application station in the South Saskatchewan River are not known. In this report, some of these environmental determinants for the effectiveness of BTH-14 were assessed.

The objectives of the project were as follows:

i) To examine the naturally-occurring aquatic bacteria in the South Saskatchewan River in mid-June to August, for production of proteolytic enzymes which might degrade the BHT-14 toxin and therefore decrease its larvicidal efficacy.

ii) To determine the distribution of BTH-14 spores, vegetative cells and delta-endotoxin in the river system.

iii) To examine whether insect death is due to BTH-14 or other infectious bacterial agents.

iv) To determine the extent of spore recycling that occurs within the cadavers of BTH-14 treated larvae at stations along the river after point application of BTH-14.

v) To collect samples and preserve these under refrigeration for a future quantitative assessment of the persistance (or dilution) of BTH-14, or its genetic material in the aquatic environment over time and distance using DNA-probe technology.

3. Experimental methods.

i) Station description.

Figure 1A shows the site of BTH-14 application and the 5 sampling stations downstream from the application site. The river depth and width varied from one to the next station and at times it was over 2 meters deep (Fig. 1B). At each sampling station, 3 metal rods (1.5 m in length) were marked and pounded into the river bed. One of the metal rods was positioned in the center of the river while the other two rods were approximately 10-20 m from either shoreline.

ii) Application of BTH-14.

BTH-14 (66.2 liters; Teknar, Lot No. 1140549, Zoecon, Dallas, Texas) was applied to the South Saskatchewan River (Birch Hills ferry station) to a concentration of 1.5 ppm. The method of application was as follows. Two individuals boarded a small outboard motor driven boat. One of the individuals drove the boat back and forth across the river, perpendicular to the flow of the river. The other individual poured 4.5 containers of Teknar (each container had 20 liters of BTH-14 formulation), into the river over a 15 min time period. At intervals, oranges were thrown into the river as markers for the BTH-14 bolus front.The flow rate of the river water on the application date was estimated to be approximately 3 meters/sec.

iii) Sample collection.

Before the BTH-14 was poured into the river, water samples were taken at each of the 5 stations, at each of the 5 sites and at 3 different positions at the site; position 1= surface of the river, position 2=middle depth of the river, position 3=bottom of the river. On location samples were

collected (Figure 2) in 15 ml sterile screw cap centrifuge tubes which were labelled according to station, site, position and sampling time. Therefore, for each time sampling period 9 water samples were taken at each of the 5 stations. The time sampling periods were 0, 1, 3, 5, 24 and 48 hours after pouring of the BTH-14 into the river at the Birch Hills ferry site.

At each station, 8 polystyrene strips (approx. 1 meter length each) were attached to the metal posts (i.e. 3 per station). The strips were attached to the metal posts 4 days prior to the BTH-14 application thus allowing blackfly larvae to colonize the strips. The density of blackfly larvae for each station, prior to the BTH-14 treatment, was estimated by removing 2 of the strips and placing them in 95% ethanol (Figure 3). The samples were brought back to the laboratory and the number of blackfly larvae counted. The reduction of blackfly larvae 48 hours after BTH-14 application was estimated by removing 2 more polystyrene strips at each station and similarly placing them in ethanol and counting the number of blackflies. Although the ethanol treatment alone killed the blackfly larvae, larve which had been dead prior to their collection were distinguishable by their blackened appearance.

iv) Plating procedures.

NPP plates (0.5% nutrient broth, 0.8% NaCl, 1.25% gm agar, 4 ppm penicillin G, 2.5 ppm polymixin B) were used for selective estimation of the number of Bacilli samples obtained from the river. NPP plates were selective in that they reduced the number of river water bacteria other than Bacillus spp.

Either 0.1 or 0.2 ml aliquots of river water were spread onto NPP plates, in triplicate. Plates were incubated at 37°C for 48 h and the total

number of bacterial colonies was enumerated. Each colony was microscopically examined to determine if it resembled the colonial characteristics of *B. thuringiensis*.

v) Drift net protocol.

Drift nets were used to sample the number of dead blackfly larvae free-floating in the river. Samples were taken at each site, 0, 24 and 48 hours after application of BTH-14. Samples were preserved in 95% ethanol and the number of dead larvae were counted.

vi) Determination of BTH-14 in blackfly larval gut.

Samples of blackened larvae found on the polystyrene strips, 48 hours post-application of BTH-14, were speciated and sorted according to instar. Groups of larvae were then surface-sterilized with 95% ethanol, washed with sterile distilled water and homogenized in phosphate buffered saline. Samples of the homogenate were diluted and plated onto NPP plates to determine the numbers of BTH-14 cells. In order to determined the number of BTH-14 spores the homogenate was heat-shocked (80°C for 10 minutes), a process which is used to kill cells but spares the spores, then plated onto NPP plates. These plates were incubated at 37°C for 48-72 hours and the number of *B. thuringiensis* colonies were counted.

vii) BTH-14 recycling and efficacy toward mosquito larvae.

Several *B. thuringiensis* isolated from dead blackflies were assessed for their larvicidal activity by their ability to cause death of the larvae *Aedes aegypti* in a laboratory bioassay. Other, non-*B. thuringiensis* spp. isolated from the river were also assessed for their larvicidal activity. *A. aegypti*

were incubated in a suspension of these bacteria and mortality was recorded over a 12 hour period. The positive controls were commercial BTH-14.

viii) Proteolytic bacteria and effects on BTH-14 efficacy toward mosquito larvae.

Of the bacterial population found in the South Saskatchewan river, several morphotypes produced extracellular proteases and were tested for their interactive effects, i.e. changing the BTH-14 efficacy toward mosquito larvae. We postulated that proteolytic bacteria occurring in the river may degrade BTH-14 toxin thus reducing their potency, or alternatively activate the toxin, against blackfly larvae. Proteolytic bacteria were identified from river samples as isolates which produced zones of clearing on gelatin-casein agar plates.

Proteolytic bacteria were cultured on gelatin-casein liquid medium. After 48 hours of growth, the cultures were centrifuged at 3K for 15 min. The recovered (1x) supernatant or the bacterial pellet was incubated with the commercial preparation of BTH-14 in a mosquito larvae bioassay. Larval mortality was recorded for up to 48 hours.

ix) River sample storage for BTH-14 molecular probe assessment.

Subsamples of river water were fixed with formaldehyde after they were assessed for bacterial density. These samples were then stored under refrigeration until molecular probe assessment of BTH-14 positive isolates.

4. Results and Discussion.

4.1 Examination of naturally occurring aquatic bacteria from the South Saskatchewan river.

River water samples were collected 1 week prior to the BTH-14 application to determine (1) the number of bacteria and spores in the river water and (2) the number of proteolytic bacteria in the river water. The number of bacteria in the river water sample, on the average, was greater than 2×10^4 /ml, the number of *B. thuringiensis* colonies was less than 10% of this and spores of BTH-14 as measured by heat shock induction was less than 1% (varying from 0-0.5%) as measured as the number of colony forming units on nutrient agar.

Among the total bacterial population, a number of proteolytic bacteria were also found in the river water which were detected by their zones of clearing around a colony growing on casein-gelatin agar plates.

4.2 Distribution of BTH-14 spores and vegetative cells in the river after BTH-14 application at the Birch Hill Ferry site.

The distribution of *B. thuringiensis*-like organisms during the 0-48 hr time course after BTH-14 application into the river is shown in Fig. 4. The data were pooled to show the distribution of *B. thuringiensis*-like organisms throughout all 5 stations during the time course (Table 1). The *B. thuringiensis*-like organisms were further characterized by light microscopy and API-50 CHB Strips (Table 2) and approximately 90% of these colonies were *B. thuringiensis*. Highest amounts of *B. thuringiensis* cells were found after 3 hr of BTH-14 application. Thereafter the amount of *B. thuringiensis* diminished with time. The low amount of *B. thuringiensis* found at 1 h post-application probably indicates that the BTH-

14 formulation had not reached most of the sampling stations. After 3 hr the BTH-14 may have adequately dispersed throughout the river at most of the downstream sampling stations.

4.3 Blackfly mortality after BTH-14 application.

Figure 5 shows the distribution, density and mortality data for two blackfly species, at 6 river sampling stations at 0 and 48 hr post-application of BTH-14 . There was significant variation in the number of larvae attaching to the test strips at each sampling station. This simply reflects the counting variation created by the flow rate, river depth and available vegetation; factors known to contribute to blackfly larvae occurrence. However, if we take the mean over all 6 sampling stations the number of live *S. luggari* larvae before BTH-14 application was 272.43 larvae/ strip and live *S. vittatum* was 61.12 larvae /strip. The mean number of dead *S. luggari* and *S. vittatum* was 9.38 and 5.38 larvae / strip, respectively. Forty-eight hours after BTH-14 application in the river the mean number of *S. luggari* and *S. vittatum* larvae was 35.16 and 12.83 larvae/ strip. This represents a 7.75- and 4.69- fold decrease, in *S. luggari* and *S. vittatum* larvae, respectively, after BTH-14 application. In other words, the *S. luggari* and *S. vittatum* larval population was reduced by 87.1% and 78.7%, respectively, after BTH-14 application. The number of dead larvae attached to the sampling strips increased 2.38-fold for *S. luggari* but stayed approximately the same (ca. 5 larvae /strip) for *S. vittatum*. However, once dead, larvae have a tendency to detach from the sampling strip, thus the number of dead larvae attached to the sampling strip underestimates the measure of BTH-14 efficacy.

We also determined the instars of the blackfly larvae on the sampling strips. Instars 1, 2, and 3 showed significantly higher susceptibility than the preceding instars after BTH-14 application. Percent mortality was related to larval instar (Fig. 6 A and B) with the highest mortality occurring for instar 1 (32.2% mortality) and instar 2 (30.9% mortality) and the lowest mortality for instar 7 (1.8% mortality) .

To estimate the number of dead larvae floating in the river we collected drift net samples. However, we were unsuccessful in obtaining reliable counts from these samples simply because of the large amount of detached, floating aquatic flora present in the river at that time of year. We found some "naturally" dead blackfly larvae, but the numbers were quite low.

4.4 Recycling of BTH-14 obtained from blackfly larvae and the effect of proteolytic bacteria.

Dissected midguts of dead blackfly larvae collected at 48 hr post-application of BTH-14 were plated onto NPP. Colonies of *B. thuringiensis* showing spores and crystals could be identified from other bacteria. The mean number of *B. thuringiensis* in the midguts from 8 dead larvae was 7.5 (+/- 3.01 S.E.) colony forming units / larval midgut. Other midgut bacteria included *B. cereus* and *B. megaterium*. The Bacilli were bioassayed against mosquito larvae (Table 4). The *B. thuringiensis* and Teknar controls gave approx. 100% larval mortality after 12 hr. The *B. megaterium* had no effect while *B. cereus* gave 95.5% mortality. Therefore, the recycled BTH-14 still retains its larvicidal activity.

Several proteolytic bacteria were isolated from river sites (Table 3) and were bioassayed (Table 5) singly or in conjuction with *B. thuringiensis*.

None of the proteolytic bacteria or the culture supernatants from these bacteria were virulent to the larvae (Table 1). Neither the proteolytic bacteria nor the culture supernatants (without concentrating) from these bacteria, antagonized the effects of *B. thuringiensis* when bioassayed against mosquito larvae.

4.5 Molecular probes for BTH-14 identification.

River samples containing BTH-14 have been preserved with formaldehyde and frozen until molecular probes for positive *B. thuringiensis* identification can be obtained. The condition of storage of these samples allows for sustained preservation until such identification can be made.

5. Conclusions and impact.

The conclusions of this study can be summarized in the following paragraphs:

(i) There is a diverse number of bacteria, reaching densities as high as 2×10^4 /ml or 20 million bacteria per liter, in the South Saskatchewan river, during the latter part of August. There is also a variation in the density of these bacteria and this probably relates to the various physical parameters of the river (Table 6) such as depth, flow rate and surrounding vegetation. We found that vegetation alone vary significantly and permit the adherence of a small portion of the BTH-14 across the 10 km range under our study. Bacteria which produce extracellular proteolytic enzymes have been detected in the river samples. The number of *B. thuringiensis* colonies at the Birch Hills ferry site, before application of BTH-14 ranged from 0-1.5×10^3/ml. A diagram to illustrate the outcome of the spreading of BTH-14 down the river stream is shown in Figure 7. BTH-14 spreads along

the moving boundary of water and meets many obstacles. In addition it tends to sediment. The net result is that the most buoyant particles in BTH-14 formulation, which are the toxic crystals are rushed away or trapped in vegetation. The BTH-14 bacterial cells and spores are diluted by the river flow. These factors are shown to quantitatively affect efficacy of the larvicidal agent. No wonder that the abatement program never produce the theoretical maximum outcome of control of the insect larvae.

(ii) The density of *B. thuringiensis* downstream from the application site, increased, overall, 3 hr post-application of BTH-14. After this time period, the density of *B. thuringiensis* declined. There was considerable variation in *B. thuringiensis* density at the various sampling stations and again this probably reflects the heterogeous nature of the physical aspects of a river and its ecosystem.

(iii) Two main species of blackfly larvae, *Simulium luggari* and *S. vittatum* were identified in the South Saskatchewan River at, and downstream from, the Birch Hills ferry site. Forty-eight hours after application of BTH-14, a 7.75- and 4.69-fold decrease, in *S. luggari* and *S. vittatum* larvae, respectively, was observed. This represents a reduction in *S. luggari* and *S. vittatum* larval population by 87.1% and 78.7%, respectively, after BTH-14 application. The mortality of blackflies was observed up to 10 km downstream from the application site. Furthermore, the trend was for mortality to increase (as observed by a decrease in the number of live larvae per strip) the further from the application site, within our sampling distance.

(iv) Microbiological variables which may interfere with BTH-14 efficacy were examined. The proteolytic bacteria found in the South Saskatchewan River inspite of their proteases did not interfere with the efficacy of BTH-14. Whether or not this observation substantiates that which occurs in situ remains to be demonstrated. We did not find evidence for midgut bacteria of blackfly larva to antagonize the effects of BTH-14.

(v) Our data show that in the laboratory BTH-14 can successfully recycle and cause mortality in other larvae. Previous to our application of BTH-14, we observed a standing population of *B. thuringiensis* in the South Saskatchewan river. The origin of this *B. thuringiensis* may be either an endemic bacterial species or an introduced species from previous BTH-14 applications. In either case, this population may contribute to blackfly larvae mortality as the *B. thuringiensis* is being recycled. To further elucidate this relationship, the *B. thuringiensis* and blackfly larval populations need to be carefully monitored in stations where there have been no previous history of BTH-14 application, to determine whether increases in population densities of blackfly larvae are followed by epizootics caused by *B. thuringiensis*.

(c) List of papers and talks

Miranpuri, G.S., Noble, P.A., Saul, L., and Khachatourians, G.G. 1989. Factors contributing to the South Saskatchewan River (SSR) Black Fly Abatement Program (BAP) using *Bacillus thuringiensis* var. *israelensis* (TEKNAR). 37th Ann. Meet. Entomological Society of Saskatchewan. Saskatoon, Sask., October 26-27.

(d) **Expenditures**: Research Accounts will be forwarding these at the end of the month.

(e) **List of personnel and percentage of time on project.**

1. Project Director:
   Dr. George Khachatourians — 5% (10 months)

2. Participating Research Scientists:
   Dr. M.J. Bidochka — 5% (9 months)
   Dr. G. Miranpuri — 15% (12 months)

3. Technical Assistant:
   Miss. C. Morrison — 100% (2.5 months)

4. Graduate Student:
   Mr. Peter Noble — 75% (10 months)

5. Summer Casual help:
   Miss. Lisa Ramsey — 100% (2 days)

Table 1. Number of Bacillus morphotypes collected as a function of water depth from South Saskatchewan River study site

Region of water column	Numbers within morphotypes		
	A	B	C
Surface	102	14	2
Middle	185	14	6
Bottom	54	45	13

Table 2. Differential characteristics of Bacillus morphotypes collected from South Saskatchewan River and known species

Characteristics	Reference strains[a]				River Isolates		
	B.c.	B.s.	B.t.i.	B.t.k.	A	B	C
Parasporal crystals	-	-	+	+	+	+	+
Pink spores	+	+	+	+	+	+	+
Glycerol	-	-	+	+	+	+	+
Erythritol	-	-	-	-	-	-	-
D-arabinose	-	-	-	-	-	-	-
L-Arabinose	+	-	-	-	-	-	-
Ribose	+	-	+	+	+	+	+
D-Xylose	+	-	-	-	-	-	-
L-Xylose	-	-	+	+	+	+	+
Adonitol	-	-	+	+	+	+	+
B-Methyl-xyloside	-	-	-	-	-	-	+
Galactose	-	-	-	-	-	-	-
D-Glucose	-	-	-	-	-	-	-
D-Fructose	-	-	-	-	-	-	-
D-Mannose	-	-	-	-	-	-	-
L-Sorbose	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-
Inositol	+	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-
a-Methyl D-mannoside	-	-	-	-	-	-	-
a-Methyl D-glucoside	-	-	-	-	-	-	-
N-Acetyl-glucosamine	+	-	+	+	+	+	+
Amygdalin	-	-	-	-	-	-	-
Arbutin	+	+	+	+	+	+	+
Esculin	+	+	+	+	+	+	+
Salicin	-	-	+	+	-	+	-
Cellobiose	+	-	+	+	-	+	+
Maltose	+	-	+	+	+	+	+
Lactose	+	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-
Saccharose	-	-	-	-	-	-	-
Trehalose	+	-	+	+	+	+	+
Inuline	-	-	-	-	-	-	-
Melezitose	-	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-
Starch	-	-	+	+	-	+	+
Glycogen	-	-	+	+	-	+	+
Xylitol	+	-	-	-	-	-	-
B-Gentiobiose	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-
D-Lyxose	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-
D-Fucose	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-
Gluconate	+	+	-	-	-	-	-
2-Keto-gluconate	+	-	-	-	-	-	-
5-Keto-gluconate	-	-	-	-	-	-	-

[a] B.c.: B. cereus, B.s.: B. sphaericus, B.t.i.: B. thuringiensis var. israelensis, B.t.k.: B. thuringiensis var. israelensis.

Table 3. Numbers of total bacteria and proteolytic morphotypes collected as a function of water depth.

Region of water column	Numbers of bacteria $x10^2$ per mL	
	Proteolytic	Total
Surface	15.0	140.0
Middle	5.0	39.0
Bottom	3.7	64.0

Colony forming units obtained if samples were plated within 6 hours of collection.

Table 4. Efficacy of commercial Bacillus species and morphotypes collected from South Saskatchewan River study site in laboratory larvicidal bioassays.

Bacillus spp. or isolate number	Hourly larval mortality 3	6	9	12 (hrs)	Larval Mortality (%)
BTH-14#1	10	10	12	15	39.5
BTH-14#2	19	34	36	36	87.9
BTH-14#3	32	32	34	34	100.0
B. cereus	0	0	11	21	95.5
B. megaterium	0	0	0	0	0
BTH-14# Blackfly gut	1	2	2	2	10.0
BTH-14#Teknar	42	43	43	43	100.0

Table 5. Killing characteristics of Bacillus morphotypes collected from South Saskatchewan River and BTH-14 in the presence of proteolytic bacterial supernatants.

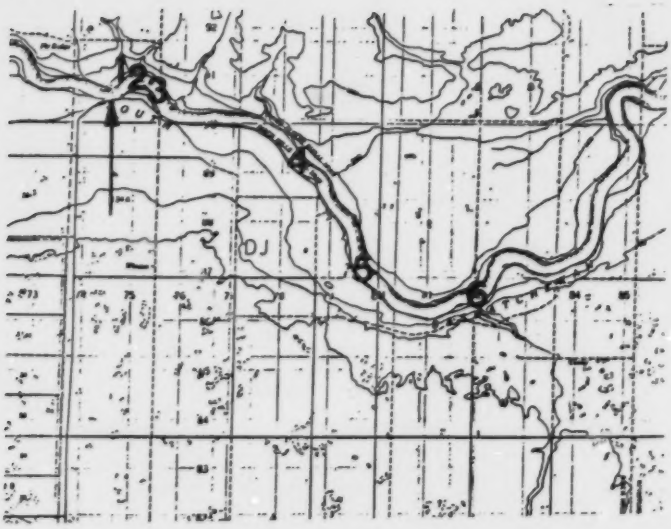
Time Post-exposure(hr)	Isolate A- Sup.	Iso A Sup	Bti+ Iso A- Sup.	Bti+Iso A- Sup.	Iso.B Sup.	Iso.B Sup	Bti+Iso B-Sup.	Bti+Iso B-Sup.
	R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2
1h	0.00	0.00	100.00	100.00	0.00	0.00	100.00	100.00
3h	0.00	0.00	100.00	100.00	0.00	0.00	100.00	100.00
5h	0.00	0.00	100.00	100.00	0.00	0.00	100.00	100.00
7h	0.00	0.00	100.00	100.00	0.00	0.00	100.00	100.00
12h	0.00	0.00	100.00	100.00	0.00	0.00	100.00	100.00
24h	0.00	0.00	100.00	100.00	0.00	0.00	100.00	100.00
36h	0.00	0.00	100.00	100.00	0.00	0.00	100.00	100.00
48h	0.00	0.00	100.00	100.00	0.00	0.00	100.00	100.00
	Iso.A cells	Iso.A cells	Bti+Iso A-cells	Bti+Iso A-cells	Iso.B cells	Iso.B cells	Bti+Iso B-cells	Bti+Iso B-cells
	R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2
1h	0.00	0.00	100.00	100.00	0.00	0.00	100.00	100.00
3h	0.00	0.00	100.00	100.00	0.00	0.00	100.00	100.00
5h	0.00	0.00	100.00	100.00	0.00	0.00	100.00	100.00
7h	0.00	0.00	100.00	100.00	0.00	0.00	100.00	100.00
12h	0.00	0.00	100.00	100.00	0.00	0.00	100.00	100.00
24h	0.00	0.00	100.00	100.00	0.00	0.00	100.00	100.00
48h	0.00	0.00	100.00	100.00	0.00	0.00	100.00	100.00
	Bti	Bti	H_2O	H_2O	Casein +Gelatin	Casein +Gelatin		
	R_1	R_2	R_1	R_2	R_1	R_2		
1h	100.00	100.00	0.00	0.00	0.00	0.00		
3h	100.00	100.00	0.00	0.00	0.00	0.00		
5h	100.00	100.00	0.00	0.00	0.00	0.00		
7h	100.00	100.00	0.00	0.00	0.00	0.00		
12h	100.00	100.00	0.00	0.00	20.00	5.00		
24h	100.00	100.00	0.00	0.00	35.00	10.00		
48h	100.00	100.00	0.00	0.00	35.00	10.00		

Table 6. Physical characteristics of South Saskatchewan River study site

Site	Distance from innoculation site (Km)	Approx. water depth (cm)	Flow rate (m/s)[a]	Dominant substrate	Weeds upsteam of site
1	0.26	50-70	0.323	rocks	no
2	0.50	40-80	-[b]	sand	yes
3	1.00	30-40	0.488	rocks	yes
4	3.25	90-100	0.311	sand	yes
5	7.25	80-100	0.397	sand	yes
6	10.25	50-100	0.352	rocks/sand	yes

a: metres/second; b: not determined

A



B

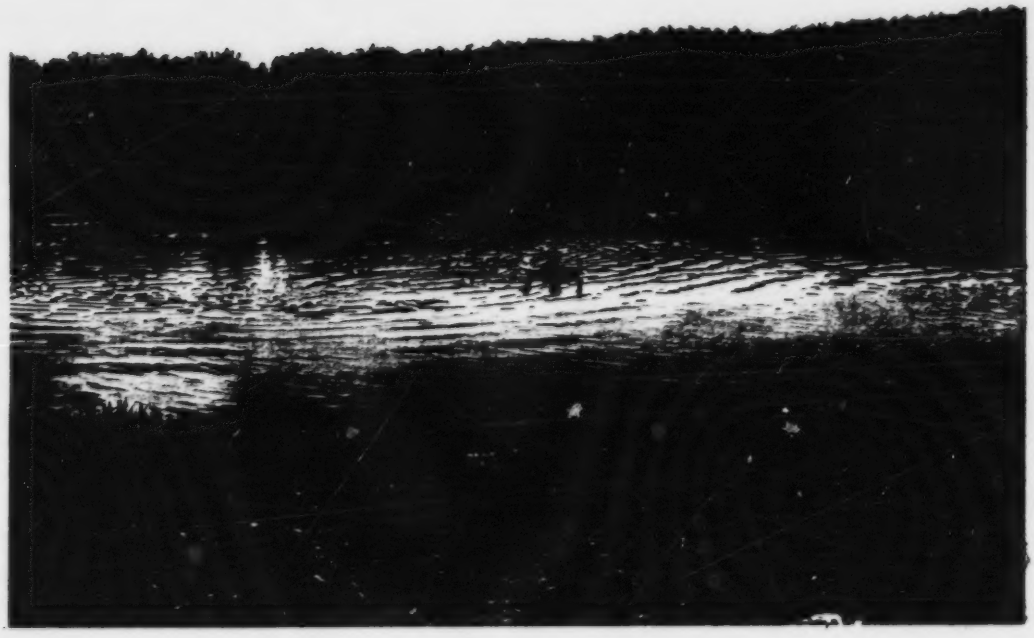


Figure 1. (A) The 6 sampling sites on the South Saskatchewan River downstream from the Birch Hill's ferry site (shown by the arrow). (B) A field researcher crossing the river at site 3.

Figure 2. Method of collecting river water samples.

Figure 3. Method of collecting and depositing polypropylene strips to which blackfly larvae attach into a jar containing 95% ethanol.

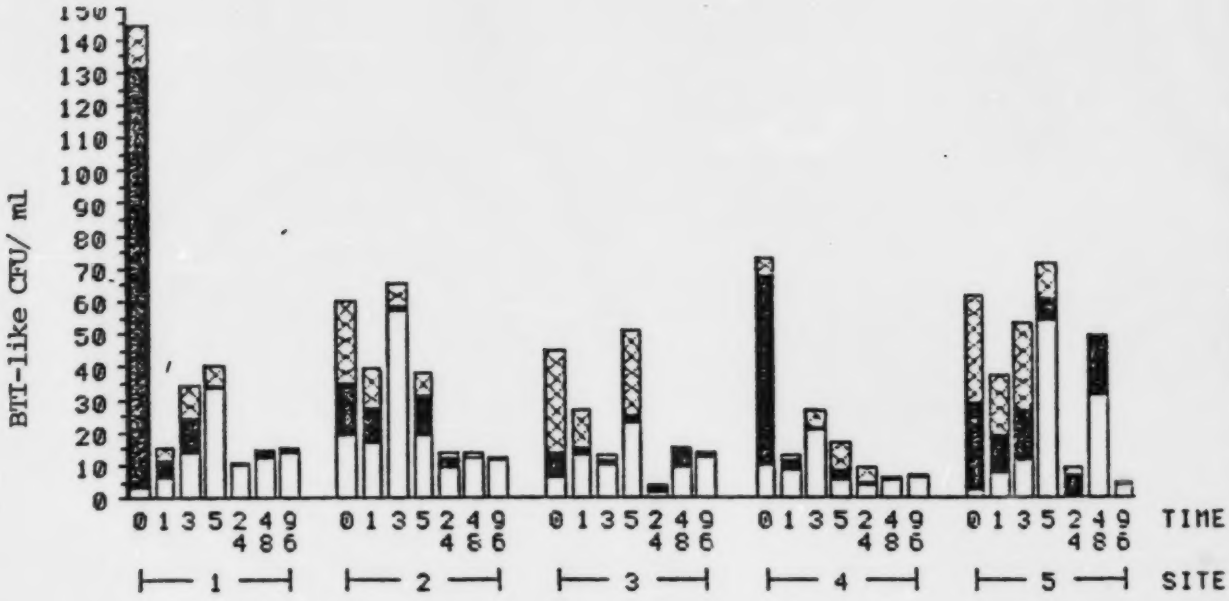


Figure 4. The number of BTH-14 like bacteria in sample counts from three water columns; sediment layer (open bars), middle layer (black bars) and top layer (hatched bars) in sites 1- 5 immediately- and at 1, 3, 5, 12, 24, and 48 hours after the application.

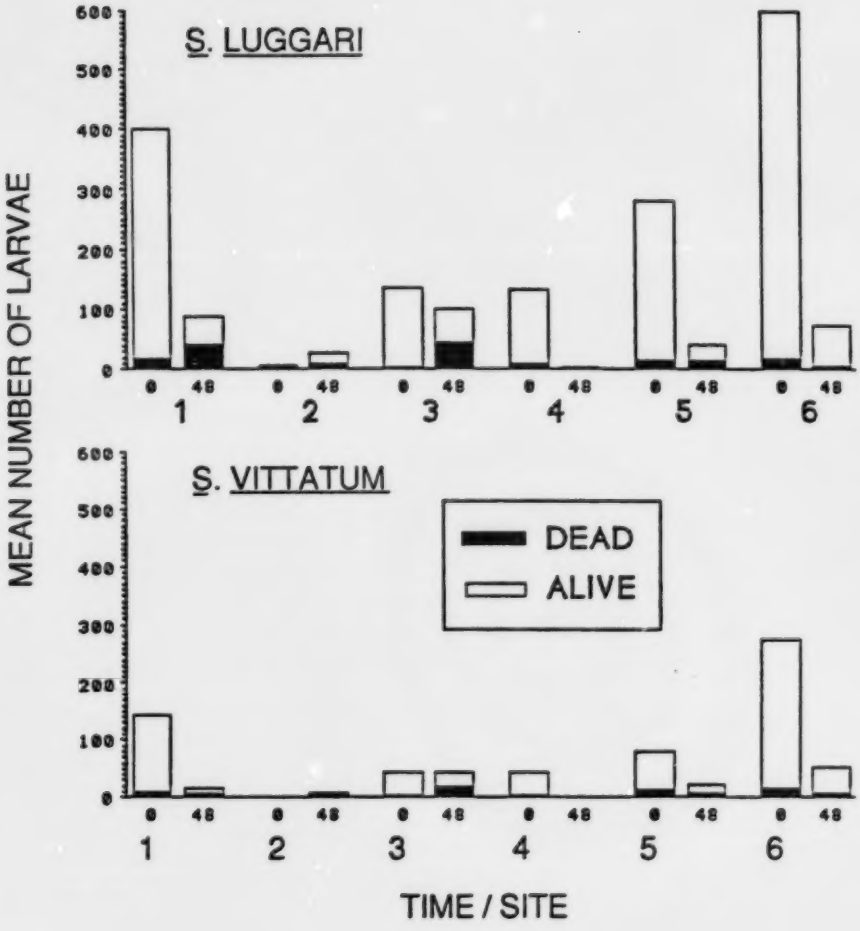
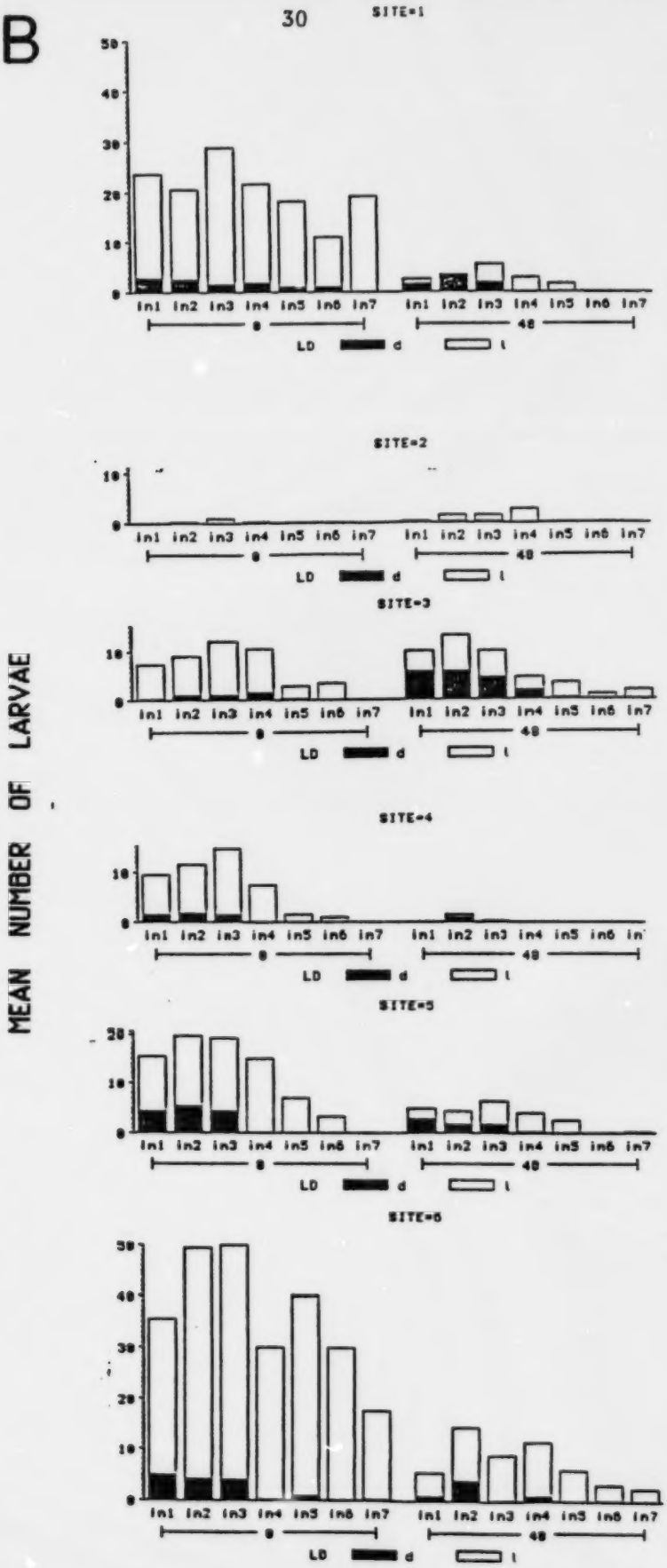


Figure 5. The number of two species of blackfly larvae (dead or alive) collected down stream from BTH-14 spray at sites 1-5, immediately or after 48 hours of application.

Figure 6. The number of *S. luggari* (A) and *S. vittatum* (B) blackfly larvae (dead or alive) and categorized by instars, collected down stream fromBTH-14 spray at sites 1-5, immediately or after 48 hours of application.

B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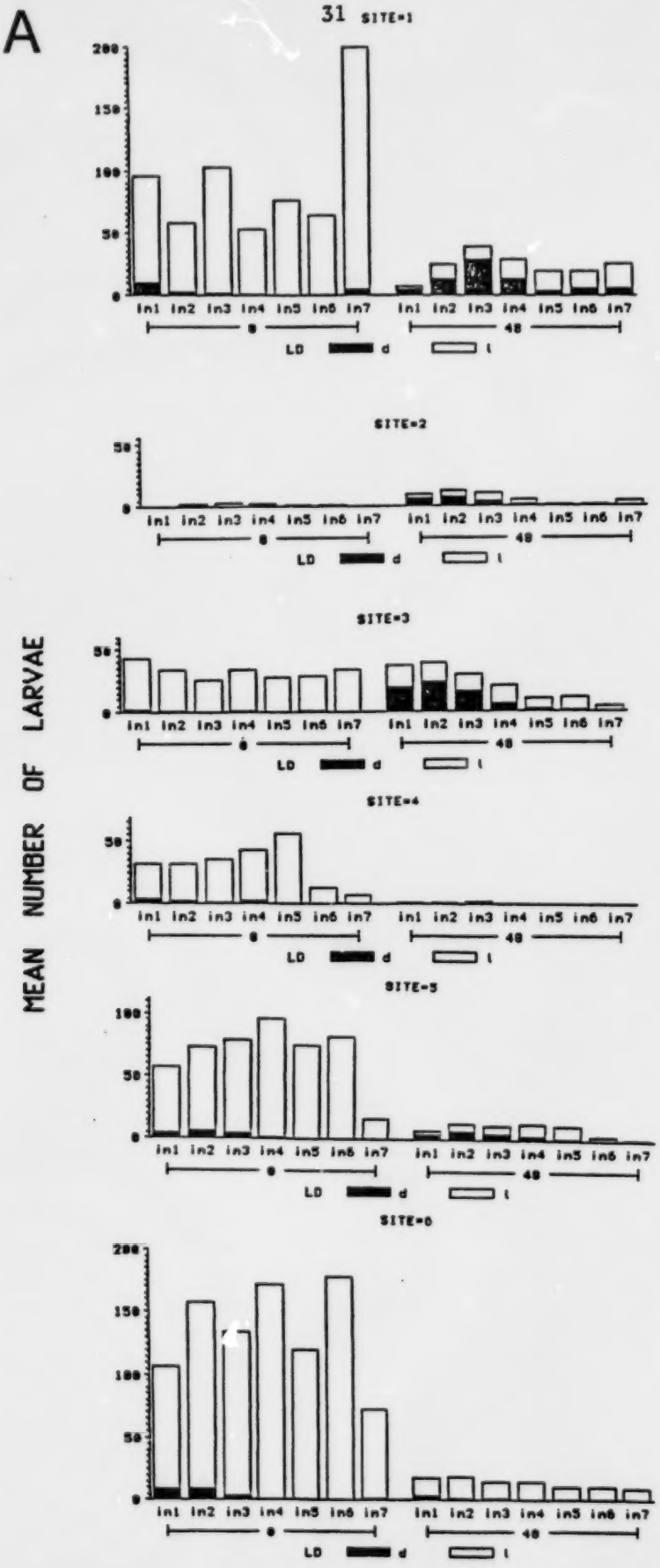
48
LD
d
l

A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48
LD
d
l
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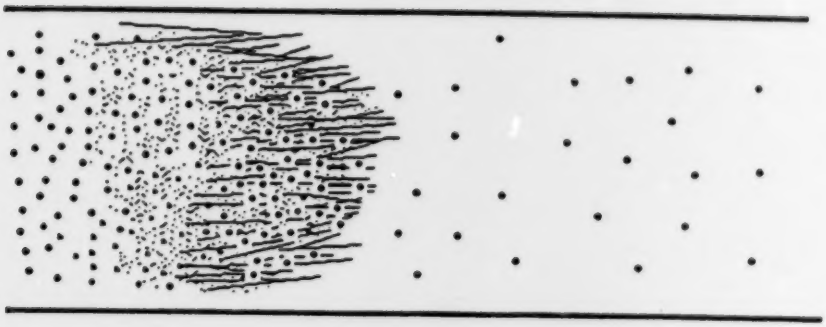
SITE=6
MEAN NUMBER OF LARVAE

HYPOTHESIS 1:



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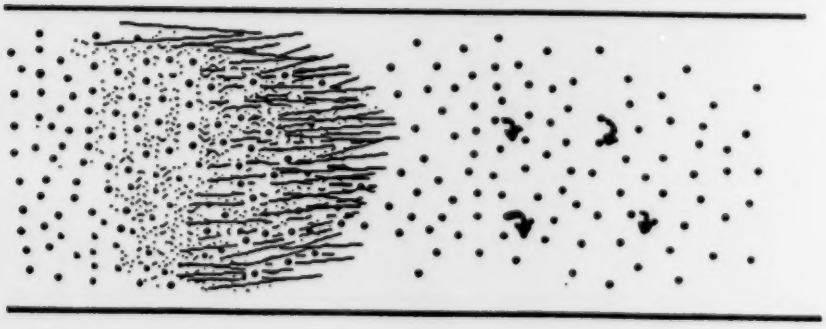

Figure 7. Possible methods by which the concentration of BTH-14 sprayed at sites 1 is distributed in the river system. Two hypotheses are suggested to account for the reduction, (1) the flow of water and presence of vegetation or other trappings and (2) the flow of water and sedimentation immediately after the application.